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PROCESS FOR THE PRODUCTION OF MAMMALS WITH DEFINED GENETIC PROPERTIES

The invention concerns the production of mammals with defined genetic properties, particularly the production of transgenic animals.

Transgenic animals are organisms in whose germ line permanent genetic variations have been introduced; a newly introduced gene is designated a transgene. Transgenic animals represent an indispensable tool for modern biology to analyze the tissue-specific regulation of genes and their function during development and in disease. Transgenic technology also offers the possibility of making available animal models for human disorders as well as producing large quantities of proteins in farm animals.

In the technique used most frequently up to now for the production of transgenic animals, recombinant DNA is microinjected into fertilized eggs; another technique in order to introduce genes into animal embryos utilizes viruses, for the most part recombinant retroviral vectors (see the review article by Wagner and Keller, 1992).

The third and most recent technique for introducing foreign genetic material into animals makes use of the potential of embryonic stem cells (ES cells) to create chimeric animals. Mammalian embryos have the capability of being able to incorporate foreign cells during their development. Two different pre-implantation embryos, usually morulae, are aggregated *in vitro*; this produces

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a chimeric embryo, which represents a mixture of both embryos. These embryos are then transferred to a pseudopregnant mouse mother, which functions as the asexual mother; the chimeric progeny obtained have a different number of cells, which originate each time from one of the two original embryos, in their tissues. The combination of these methods with the use of ES cells has been proven very effective in producing genetically manipulated animals.

Embryonic stem cells are derived from the inner cell mass ("inner cell mass", ICM) of blastocysts; they are totipotent cells, which can develop into all cell lineages, including germ cells, if they are introduced into an embryo by injection into diploid blastocysts or by aggregation with morulae (Robertson, 1987; Bradley, 1987; Beddington and Robertson, 1989; Nagy et al., 1990). ES cells can be isolated from blastocysts and then established as permanent cell lines, if they are cultured under well-defined culture conditions that are precisely maintained; they may be genetically manipulated. On the basis of this capacity, they represent an effective tool for modification of the mammalian genome, particularly the mouse genome, by introducing targeted mutations or other genetic variations into animals with their help (Wagner et al., 1991; Ramirez-Solis et al, 1993; Skarnes, 1993; Bronson and Smithies, 1994).

For some time, cells of the designation "embryonic germ cells" ("EG cells") have been available, which can be cultured from primordial germ cells to immortalized cell lines, and are similar to ES cells in many respects; and among other things, EG cells are totipotent, and they can be manipulated like ES cells

and form germ-line chimeras, if they are introduced into blastocysts (Donovan et al., 1997).

In the course of the last few years, different experimental techniques have been developed in order to produce animals, which are derived from totipotent cells. (Totipotent cells are cells with the capability of differentiating to all somatic cells as well as to germ cells.) In the case of ES cells, these methods primarily have the objective of obtaining the entire development potential of ES cells *in vitro* (Williams et al., 1988; Smith et al., 1988) and to limit the development potential of host cells in the case of formation of chimeras and thus to increase the frequency of formation of germ-line chimeras (Nagy et al., 1990; Kaufman and Webb, 1990). One of the most important advances in the development of these techniques is the use of tetraploid embryos as host cells, since tetraploid cells have only a limited potential for development after implantation (Nagy et al., 1990; Kaufman and Webb, 1990; Kubiak and Tarkowski, 1985). If tetraploids are aggregated with diploid embryos, the differentiation of tetraploid cells is extensively limited to the primitive endoderm and the trophectoderm, which consequently form extraembryonic tissue, whereas diploid cells can form the actual embryo (James and West, 1994; James et al., 1995).

In an earlier study, various ES cell lines were aggregated with morulae in order to produce fetuses in this way, which originate completely from ES cells (organisms originating completely from ES cells are designated in the following as "ES animals", e.g. "ES mice", or "ES fetuses"); the ES fetuses that are obtained, however, perish at birth (Nagy, 1990). Further studies showed that

viable and fertile ES mice originating exclusively from ES cells can be obtained, if wild-type R1 cells of an early passage (Nagy et al., 1993) or TT2 cell lines (Ueda et al., 1995) are used for the aggregation with tetraploid morulae.

ES mice were also produced by injecting ES cells into diploid blastocysts in a first step, whereby chimeric mice were first obtained; further crossings produced ES mice after two generations. The method of injection into blastocysts was first described by Gardner, 1968, and a simplified version was described by Bradley and Robertson, 1986, as well as by Bradley, 1987.

The objective of obtaining viable ES mice by use of ES cells from later passages has not been achieved with previously available methods (Nagy et al., 1993); the production of ES mice with the use of genetically modified ES cells in general appeared impossible. (The applicability of ES cells from later passages is particularly of importance with respect to the use of cell lines, further with respect to the use of genetically modified ES cells, whose selection, of course, is accompanied by an increase in the number of passages.)

The object of the present invention was to provide a new process, with which mammals with defined genetic properties, particularly transgenic mammals, can be obtained, which are completely derived from totipotent cells.

The object set forth above is solved by a process for producing mammals with defined genetic properties, particularly transgenic mammals, in which totipotent cells of the same mammalian species are introduced into blastocysts and the obtained embryo is transferred to an asexual mother. The process is

characterized by the fact that totipotent cells with defined genetic properties are introduced into tetraploid blastocysts.

Animals, which are derived completely from totipotent cells, are obtained by means of the process according to the invention. The process of the invention offers the advantage of obtaining animals completely derived from totipotent cells in a single step from *in-vitro* cultured totipotent cells (ES cells or EG cells).

According to definition, animals which bear a permanent genetic modification of any type whatever fall under the term "transgenic mammals" within the scope of the present invention,.

Animals, which are "completely derived from totipotent cells", preferably contain up to 100% cells that are derived from ES cells or EG cells. However, the animals may also have a small proportion, preferably no more than 10%, of cells that are derived from the tetraploid blastocysts.

In a preferred form of embodiment of the invention, the mammals are mice; the process, however, can be applied basically to all mammals, from which ES cells or EG cells can be obtained. The prerequisite for obtaining totipotent cells from mammals other than mice is the definition of conditions, which permit the culture of ES cells or primordial germ cells from these organisms and the establishment of ES or EG cell lines, including the requirement of specific growth factors as well as feeder cells for co-culture with ES cells or EG cells. These conditions may be determined empirically by means of serial experiments.

The isolation of ES cells from blastocysts, the establishment of ES cell lines and their subsequent culture are undertaken according to conventional

methods, such as described, e.g., by Doetchman et al., 1985; Li et al., 1992; Robertson, 1987; Bradley, 1987; Wurst and Joyner, 1993; Hogan et al., 1994; Wang et al., 1992. The culture of EG cells can be conducted according to methods described in the review article by Donovan et al., 1997, as well as in the original literature cited therein. Totipotent cell lines, e.g., mouse ES cell lines, can be tested in preliminary experiments as to whether they are suitable for use within the scope of the present invention, based on their developmental potential. For this purpose, cells of the lines of interest can be injected into diploid mouse embryos, the obtained embryos can be introduced into asexual mothers, and the young animals can be investigated for rate of chimera formation as well as for the frequency of formation of germ-line chimeras.

In a preferred form of embodiment of the invention, the totipotent cells are ES cells.

Tetraploid blastocysts can be obtained according to known methods by electrofusion of two-cell embryos and then cultured, as described, e.g., by James et al., 1992; Nagy and Rossant, 1993; or by Kubiak and Tarkowski, 1985.

The introduction of ES cells or EG cells into blastocysts is also conducted in a manner known in and of itself. The method of microinjection, as described, e.g., by Wang et al., 1991, is preferred within the scope of the present invention. In the case of conventional microinjection, approximately 5-10 ES cells, which have been removed from a single cell suspension in a micromanipulation apparatus, are injected into a blastocyst, which has been immobilized by means

of a holding pipette. Then the embryos are incubated for at least 3 h, and overnight, if necessary.

In a preferred form of embodiment of the invention, genetically manipulated totipotent cells are used to obtain transgenic animals.

There are no limitations with respect to the type of genetic variation of totipotent cells: genes can be overexpressed, mutated or, with respect to the production of so-called "knock-out" animals, may be excluded; further, foreign genes can be inserted or intrachromosomal deletions can be made. The genetic variation can be undertaken on one or both alleles, the latter approach being described, e.g., by Hilberg and Wagner, 1992, for the exclusion of the c-*jun* gene. The fact that the present invention makes possible a genetic change on both alleles is a particular advantage; it was only possible with methods of the prior art to obtain transgenic animals, in which both alleles had the desired modification after additional crossings and protracted breeding of animals that had a genetic modification on one allele.

Conventional methods can be used for the genetic manipulation of totipotent cells. Generally plasmids, preferably linearized plasmids, which bear the desired genetic modification, are used. With respect to the selection capacity of the genetically modified ES cells, the plasmids appropriately contain a marker gene, e.g., neomycin, hygromycin or puromycin resistance gene, under the control of a promoter. With respect to the expression of a gene contained on the plasmid in the host cells, the plasmid may contain gene-expression control

sequences, e.g., a strong promoter, such as PGK (phosphoglycerin kinase) promoter, which is functional in ES cells or EG cells.

The methods by means of which the plasmid is introduced into cells are standard methods for *in-vitro* transfer of DNA in mammalian cells, which are known from the literature, e.g., electroporation, calcium phosphate precipitation, or methods based on receptor-mediated endocytosis, disclosed, e.g., in WO 93/07,283.

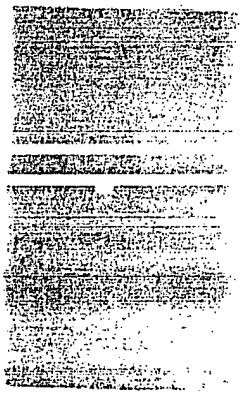
Another method for introducing genetic modifications into totipotent cells utilize viruses, e.g., recombinant retroviral vectors; what has been said relative to plasmids is basically valid with respect to sequence segments contained in the vector, which make possible the selection of genetically modified cells or expression in cells (Wagner and Keller, 1992; Stewart et al., 1985).

It is possible by means of the process according to the invention to produce viable and fertile transgenic mammals, particularly ES or EG mice in a routine manner from *in-vitro* genetically modified totipotent cells.

Transgenic animals, which can be used, among other things, for studies of gene function or for the production of proteins, can be produced in a reproducible manner by means of the process of the invention, among other things from genetically manipulated totipotent cells, which overexpress, e.g., a specific gene, or in which a specific gene has been inactivated. The process according to the invention, when compared with conventional methods for producing transgenic animals, offers a powerful, rapid and economical possibility for producing mutant

animal fetuses, particularly mouse fetuses, as well as transgenic strains directly from totipotent cells, in which the desired genetic changes have been made.

All three ES cell lines of the designations D3, R1 and GS1 investigated within the scope of the present invention formed germ-line chimeras after injection into diploid blastocysts. In the case of injection into tetraploid blastocysts, living ES mice were obtained from R1 and GS1 cells. Living ES mice could not be successfully produced with D3 cells, even with the use of cells of an early passage (passage 9), after injection of ES cells into tetraploid blastocysts. This agrees with earlier observations from aggregation experiments (Nagy et al., 1990; see also Table 2) with these cells. The inability of D3 cells to form viable ES mice is probably not to be attributed to the fact that these cells have lost their development potential; D3 cells already have been used frequently in so-called "gene-targeting" experiments, wherein a high rate of formation of chimeras and germ-line chimeras could be obtained after their injection into diploid blastocysts (Urbánek et al., 1994; Wang et al., 1992; Wang et al., 1994; see also Table 1). However, it is possible that the potential of D3 cells to differentiate into several cell types that are critical for the adaptation of the fetus to postnatal life is adversely affected due to unknown genetic or epigenetic changes. This assumption is supported by the observation that D3 cells are in a position to produce fetuses that develop up to the normal birth term, but that the newborn animals are not in a position to maintain respiration, as well as the fact that they have an increased body weight and polydactyly and perish at birth. These properties are reminiscent of the phenotypic features of



mice in which the "imprinted" Igf2/Mpr gene is missing (Wang et al., 1994; Lau et al., 1994); it may happen, therefore, that imprinted genes or genes that regulate the growth of fetuses are responsible for the observed effect. Whereas in the environment of the host cells of diploid embryos, faulty functions of ES cells should be complemented by the host cells, the intrinsic developmental potential of D3 cells to be able to form all functional cell types, may be limited in an environment derived completely from ES cells due to the inability of D3 cells to differentiate. The introduction of various wild-type ES cells into tetraploid embryos, appropriately in serial experiments, can thus serve as a rapid and reliable test to check the suitability of ES cells for use within the scope of the present invention.

The genetic background of the respective mouse strains, from which the various ES cells originate, should be another factor that influences the viability of the ES mice. All of the ES cell lines used within the scope of the present invention were derived from mouse strain 129: The R1 cells were derived from a mouse blastocyst from a cross between the substrains 129/Sv and 129/Sv-CP (Nagy et al., 1993); GS1 cells were derived from 129/Sv/Ev. D3 cells (Doetchmann et al., 1985) and J1 cells (Li et al., 1992) were derived from 129/Sv or 129/terSv. TT2 cells, which also produce ES mice, were derived from a hybrid strain F1 (C57BL/6 x CBA) (Yagi et al., 1993). Based on the results obtained within the scope of the present invention as well as earlier studies (Nagy et al., 1993, Ueda et al., 1995), it cannot be excluded that ES cell lines, which are

derived from different mouse strains or substrains, have different capacities for forming viable ES mice.

The efficiency in the production of newborn ES mice by means of injection of wild-type R1 cells into tetraploid embryos (14%) was higher than production by means of aggregation (6% within the scope of the present invention or 7% in the study described by Nagy et al. (1993)). This result is in agreement with a comparison between the methods of aggregation and injection of ES cells into diploid embryos (Wood et al., 1993). The use of tetraploid blastocysts according to the invention for injection methods resulted in the fact that a few selected R1 cell clones, which had been cultured *in vitro* for longer than 24 passages (e.g., R169.2.3 and R-*fra*3), still have the capacity of producing viable ES mice. These results are noteworthy, particularly in view of the results of earlier aggregation experiments, in which wild-type R1 cells lost their capacity to produce viable ES mice after passage 14 (Nagy et al., 1993). The reason why the injection of ES cells into tetraploid blastocysts leads reproducibly to the formation of ES mice is not completely clarified. Since ES cells are obtained originally form ICM of blastocysts and these ICM cells are also very similar (Beddington and Robertson, 1989), it is conceivable that both the spatial vicinity of ES cells and ICM as well as also their compatibility in development are responsible for this effect. This assumption is also supported by the observation from comparative experiments that the efficiency of producing chimeric mice was smaller, if ES cells were introduced into diploid morulae under the "zona pellucida", than when the

conventional blastocyst injection method (injection into diploid blastocysts) was used.

The high efficiency of the method according to the invention makes it superior when compared to methods of the prior art (aggregation of ES cells with tetraploid blastocysts or injection into diploid blastocysts) and offers the only possible method at the present time for generating mutant mice directly from genetically modified totipotent cells.

The generation of viable mutant mice directly from genetically manipulated totipotent cells has many advantages. Since the fetal tissues derive completely from totipotent cells that can be genetically modified, this technique offers a direct way of producing fetal material of pure ES or EG cell origin for cell-biological, molecular-biological or genetic studies (Forrester et al., 1991; Carmeliet et al., 1996).

ES fetuses reproduce the expression patterns of specific genes, such as, e.g., the *Pax*5 gene or a "trapped" gene, in a reliable manner, in comparison with fetuses that are derived from the same ES cells by crossings of heterozygous mutant mice. Advantageously, ES fetuses can be used for expression studies, since a rapid production of fetal material (several days) is made possible thereby, whereas conventional breeding normally requires four to five months. In addition, the reliable and reproducible expression pattern in ES fetuses minimizes possible complications in conventional chimeric tissues, which, according to definition, are comprised of both wild-type as well as mutated ES cells. This technique is thus useful for studies of gene function or for

identification of new genes, e.g., in "gene-trap" studies (Skarnes, 1993). It has been shown that mutant mice lines, e.g., c-*fos* transgenes and *fra*-1-"knock-out" mice can be produced directly from mutant ES cells in an efficient manner by means of the method according to the invention (injection of ES cells into tetraploid blastocysts). The process according to the invention offers the possibility of producing transgenic mouse lines from ES cells or EG cells, which are preselected for the integration and expression of transgenes. The efficiency of producing mice that overexpress a specific gene is significantly improved thereby – in comparison with conventional injection methods, in which diploid blastocysts are used. The process according to the invention has been applied in several gene-overexpression and gene-inactivation studies.

In addition, it is possible to produce mutant tissue for the study of specific effects by this method, if inactivation or overexpression might lead to death or to adversely affected gametogenesis in heterozygous mutants or even in chimeras (e.g., Carmeliet et al., 1996).

Lastly, the process according to the invention offers the possibility of rapidly and economically producing mutant animal strains, particularly mouse strains, and to have rapid access to mutant fetuses and animals, which represents a basic advantage for research in the field of mouse genetics.

In addition to the production of transgenic animals, the process of the invention can be used for the production of genetically unmodified ES animals or EG animals, which have specific desired properties. For this purpose, totipotent cells are used, which are preselected relative to the desired properties by culture

or breeding experiments, in order to obtain identical animals with the respective properties.

Overview of the Figures

Fig. 1: Production of tetraploid embryos. Injection of ES cells into tetraploid blastocysts.

Fig. 2: GPI analysis of newborn ES mice and of the progeny of ES mice, that derive from R1 cells.

Fig. 3: Comparison of *lacZ* gene expression in ES fetuses and in fetuses which originate from heterozygote crossings.

In the following examples, which illustrate the present invention, the following materials and methods were used, if it is not stated otherwise:

a) Mice: C57BL/6 mice were used as the donors of diploid embryos and B6CBAF1 mice (C57BL/6 x CBA) were used as the donors of tetraploid embryos. Both strains are homozygous for the Gpi-1^b allele at the *Gpi*-1 locus, which codes for glucose phosphate isomerase (GPI).

b) ES cells and gene transfer: the following ES cells, which have been described in the literature, were used:

D3 cells (Doetchmann et al., 1985)

R1 cells (Nagy et al., 1993)

J1 cells (Li et al., 1992).

GS1 cells were isolated from blastocysts of the substrain 129/Sv/EV. This mouse strain was established from a chimeric mouse obtained after germ-line transfer of ES cells of the genotype of AB1. AB1 ES cells had been originally

estabiished from substrain 129/Sv/EV, as has been described by McMahon and Bradley, 1990, and by Papaioannou, 1993. The method described by Robertson, 1987, was essentially used for isolating the GS1 cells: blastocysts were plated onto a 4-well plate onto feeder cells, which propagated ICM after 5 days of culture. Clumps similar to ES cells were broken up with a pipette and plated out onto a new plate with feeder cells. The expanded ES cells were identified and again investigated for their karyotype and their totipotency relative to development.

All ES cells were originally isolated from the propagation of blastocysts, which were obtained from mouse strain 129, which is homozygous for the Gpi-1^a allele. In order to modify the ES cells, R1 cells of passage 16 were electroporated with various constructs; G418-resistant clones were selected and expanded prior to injection. The following constructs were used: a vector which over-expresses c-*fos* (Wang et al., 1991); a so-called "gene-trap vector" of the designation pSAβgeo, which contains one *lacZ-neo* fusion gene without promoter and has the property of being able to integrate any genes whatever into introns (Friedrich and Soriano, 1991); a so-called "gene-targeting vector", which interrupts the *Pax*5 gene by homologous recombination in mice (Urbánek et al., 1994); as well as a gene-targeting vector, which interrupts the endogenous *fra*-1 gene ("Fos-related antigen 1") by homologous recombination*. For production of the *fra*-1 gene-targeting vector, several cosmid clones that contain the *fra*-1 gene

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* Reference is omitted – Trans. note

were isolated from a genomic mouse library, and the sequence as well as the exon/intron organization of the entire gene were determined. On the basis of this information, a null mutation was introduced in the *fra*-1 gene by homologous recombination in ES cells. Proceeding from plasmid pGNA (Le Mouellic et al., 1990, 1992), a gene-targeting vector was constructed. The essential DNA binding and dimerization (leucine zipper) domains of *fra*-1 were replaced by the bacterial genes coding for β-galactosidase and neomycin resistance, which serve, respectively as a reporter gene and a selectable marker in mammals. After the electroporation of ES cells, G418-resistant colonies were analyzed either by means of Southern blot or staining for β-galactosidase actively, in order to confirm the integration of the vectors.

c) Obtaining of two-cell embryos; Electrofusion: Two-cell embryos were isolated from female B6CBAF1 mice on day 1.5 post-coitum (p.c.) and used for the production of tetraploid blastocysts (Fig. 1A). Tetraploids of embryos were produced by means of electrofusion, wherein the method described by Nagy and Rossant (1993) was modified: Two-cell embryos were equilibrated for 30 seconds in a 0.3 M mannitol solution before they were arranged individually between two platinum electrodes in 0.3 M mannitol and subjected to a short current pulse at 95 V during a period of 30 µsec in an effective field of 2 V with the use of the current-pulse generator CF-100 (Biochemical Laboratory Service, Budapest) (Fig. 1B). After an incubation time of 15 min, two blastomers began to fuse (Fig. 1C, open arrow) and to gradually form a one-cell embryo (Fig. 1C, arrow).

d) Aggregation of ES cells with morulae; injection of ES cells into blastocysts: For the aggregation, the morulae were isolated either from the oviducts of pregnant mice (day 2.5 p.c.) or obtained from tetraploid one-cell embryos by culturing these for 24 to 40 h after electrofusion. The processing and aggregation of ES cells was conducted as described by Nagy et al. (1990). Diploid blastocysts were isolated from the uterus of pregnant C57BL/6 mice (day 3.5 p.c.). In order to obtain tetraploid blastocysts, the electrofused one-cell embryos were cultured for 48 to 60 h after fusion (M16 medium at 37°C in an incubator containing 95% air/5% CO_2; Fig. 1D). Wild-type or genetically manipulated ES cells were then injected into diploid or tetraploid blastocysts according to the method described by Wang et al. (1991) (Fig. 1E, Fig. 1F). While the injected diploid embryos developed in the normal gestation period and then were naturally delivered, the pregnant mice that had received the tetraploid embryos were subjected to a Caesarian section on day 18.5 (Nagy et al., 1990). Viable fetuses, evaluated on the basis of heart beat and respiration, were removed from female that had given birth on the same day or on the previous day. Several of the living young were investigated for the GPI marker. The surviving ES mice were further paired with wild-type C57BL/6 mice in order to test them for fertility and germ-line transmission.

e) GPI isoenzyme analysis: Various tissues of fetuses or adult animals, which were derived from ES cells, were isolated and cut up into small pieces in distilled water. The samples were lysed by three freeze/thaw cycles, and, as described by Bradley (1987) and Wang et al. (1991), subjected to GPI analysis

after protein extraction. The proportion of cells that originated from ES cells or from the host was estimated in the chimeric tissues from the ratio of GPI-1A or GPI-1B isoenzyme activity, which was made visible in a coupled optical assay.

f) β-Galactosidase histochemistry: The method described by Sanes et al. (1996) was used in modified form in order to determine the β-galactosidase activity in fixed intact embryos. The embryos and their extra-embryonic membranes were fixed for 5-10 min (100 mM Na phosphate, pH 7.4, 5 mM EGTA, 2 mM $MgCl_2$, 0.2% glutaraldehyde), then washed 3 times (100 mM Na phosphate, pH 7.4, 2 mM $MgCl_2$, 0.01% Na deoxycholate, 0.02% NP-40) and stained with a histochemical reaction mixture (100 mM Na phosphate, pH 7.4, 2 mM $MgCl_2$, 0.01% Na deoxycholate, 0.02% NP-40, 5 mM K-iron(III) cyanide, 5 mM K-iron(II) cyanide and 1 mg/ml X-Gal).

Example 1

Production of chimeric mice, which originate from diploid embryos and wild-type ES cells.

In order to test the developmental potential of various ES cell lines, four different wild-type ES cell lines were first used in order to produce chimeric mice, and this was done, on the one hand, by aggregation of ES cells with diploid morulae, and on the other hand, by their injection into diploid blastocysts. It could be shown by all four ES cell lines that they are in a position to deliver a high rate of formation of chimeras after they have been introduced into diploid mouse embryos, as well as to form germ-line chimeras with high frequency (see Table 1). Interestingly, a high proportion of female chimeras were also obtained

with R1 and J1 cells, several of which transmitted the Agouti skin color of strain 129/Sv to their progeny (Table 1).

Example 2

Production of viable ES mice by aggregation of ES cells with tetraploid embryos or injection of cells into blastocysts.

In order to produce tetraploid embryos, two-cell embryos received a short current pulse, which led to the fusion of approximately 90% of the embryos. These embryos were further cultivated. Five experiments were conducted, in which the embryos developed with high frequency into morulae (68-95%) and into blastocysts (80-98%). The morulae were used for aggregation with ES cells; and the blastocysts were used to inject ES cells into them (Fig. 1E, Fig. 1F). All four wild-type ES cell lines (D3, R1, J1 and GS1) were tested with respect to production of ES mice. From the aggregation with D3 cells, 26 living newborn were obtained after Caesarian section, but not a single one of these survived (Table 2). Likewise, no viable ES mice could be obtained from J1 cells (Table 2). In contrast to this, R1 cells produced ES mice with similar frequency after aggregation with tetraploid morulae (see Table 2), as described by Nagy et al., 1993.

Since the method of injection of ES cells into diploid blastocysts relative to formation of chimeras was similarly efficient to the aggregation methods (see also Wood et al., 1993), as a next step, it was investigated whether ES mice could be obtained by injection of wild-type ES cells into tetraploid blastocysts. With the use of D3 cells from passage 9, a relatively high proportion (28%) of

fully developed fetuses could be obtained on day 18.5 p.c. (E 18.5) by means of Caesarian section (Table 2); the newborn animals, however, could not maintain respiration and perished shortly thereafter. Interestingly, these newborn animals had a higher body weight as well as polydactyly. Nine viable young animals were born by means of Caesarian section at time point E 18.5 from 36 tetraploid blastocysts, which had been injected with R1 cells (passage 14) (Table 2). Five of these could maintain respiration and were removed from an asexual mother. Regrettably, two young animals could not be found after 7 days and one perished at the age of 5 weeks. Two of these ES mice survived to adulthood and showed complete germ-line transmission (Table 2). After injection of GS1 cells into 54 tetraploid blastocysts, 17 embryos developed in the normal gestation period (Table 2). Six young animals were delivered by means of Caesarian section and could maintain respiration. Five of these perished within 48 hours, but one ES mouse survived to adulthood and transmitted the genetic material originating from ES cells to its progeny (Table 2).

Example 3

Production of ES mice with genetically modified ES cell clones

Since viable ES mice had been produced from tetraploid blastocysts, into which R1 or GS1 wild-type cells had been injected, it was next investigated whether the method according to the invention is also suitable for supplying mutant ES mice from genetically manipulated ES cells. First, R1 cells were electroporated with the c-*fos* expression vector and two G418-resistant clones of the designations R-169.2.3 and R-169.2.5 were used for injection into tetraploid

blastocysts (the R1 clones were cultured for more than 24 passages prior to their injection into blastocysts). Clone R-169.2.5 was injected into a total of 103 blastocysts; twelve newborn animals were obtained by means of Caesarian section. Three of them maintained respiration, but perished after 48 h. Clone R-169.2.3 produced a higher number of surviving newborns; 23 young were viable after Caesarian section, twelve of which were removed from an asexual mother (Table 2). Unfortunately, seven of the newborns perished in the first three days due to insufficient care by the surrogate mother. Two other mice were lost during the weaning phase. Three mice survived to adulthood. After it had been confirmed by Southern blot analysis that the transgene had been transmitted to the progeny, two transgenic lines were established.

In another experiment, an R1 clone of designation R-*fra* 3 (*fra*-1 +/-) from passage 24 was used, in which an allele of the *fra*-1 gene is interrupted by homologous recombination. R-*fra*3 cells were injected into 48 tetraploid blastocysts; eight living young were obtained by means of Caesarian section. Four of five newborn animals removed from a surrogate mother reached adulthood, and for three of these, it could be confirmed that they transmitted the mutated allele (*fra*-1+/-) to the progeny (Table 2). The female chimeric mouse was sterile, which was in contrast to the results obtained with wild-type R1 cells, in which chimeric females, which could give rise to germ-line progeny, were obtained with these cells and diploid embryos.

Example 4

GPI analysis in tissues of ES fetuses and ES mice

In order to confirm that the fetuses and adult mice obtained according to the above examples actually derived exclusively from ES cells, a GPI analysis was conducted, by means of which the contribution of ES cells to tissue formation can be determined. From the experiments, in which an aggregation had been conducted, all eleven fetuses from D3 cells, one from R1 cells and one from J1 cells showed a 100% derivation from ES cells in all of the investigated tissues (Table 3). One of the fetuses derived from R1 cells had a small proportion of tissue (approximately 10%) in the heart, which originated from host cells, but the other investigated tissues derived exclusively from ES cells (Fig. 2A, Table 3). Most of the ES fetuses, and all of the adult ES mice that were derived from R1 and GS1, which had been produced by means of injection of cells into tetraploid blastocysts, were equally exclusively derived from ES cells, with the exception of two fetuses derived from D3 (E18.5), which had a contribution of host cells to the extent of 10 to 50% in the heart, lungs and liver. It is worthy of note that four of the fetuses derived from R1 cells already showed in the early stage (day 13.5 p.c.) exclusively the ES-specific GPI-1A marker (Table 3). In addition, the progeny of ES mice deriving from R1 were investigated by means of GPI analysis, whereby it could be shown that they derive from ES cells.

ES fetuses and adult mice, which had been produced by injection of genetically modified R1 cells into tetraploid blastocysts, were also investigated by means of GPI analysis. It was shown from tissues of two newborn animals (E18.5), which were derived from the ES clone R1-169.2.5, that they derived

completely from ES cells. The GPI analysis of 3-day-old young (derived from D3 cells) and adult mice, which had been produced with R1 cells, which have either a c-*fos* transgene which is overexpressed (R-169.2.3) or an inactivated allele of *fra*-1 (R-*fra*-3), showed in all investigated tissues that the latter were derived from ES cells up to 100% (Table 3). Further, GPI and Southern blot analyses were conducted on the progeny of these ES mice. It was thus confirmed that only the GPI-1A marker was present in all progeny; several also inherited either the transgene (c-*fos*) or the interrupted allele *fra*-1. Fig. 2A shows the GPI analysis of newborn ES mice, which derived from R1 cells. All tissues, except placenta and heart, only contained the GPI-1A marker, which indicates a 100% derivation from ES cells. Fig. 2B shows the GPI analysis of the progeny of one ES mouse: the blood of four young animals from one adult ES mouse exclusively contained the GPI-1A isoenzyme, which confirms the ES derivation of the progeny.

Example 5

Comparison of the gene expression pattern of ES cell embryos and germ-line embryos

In order to test the suitability of the process according to the invention for the production of ES mice for the investigation of gene expression and mutant phenotypes, it was investigated when and where specific genes were expressed in ES cell embryos and in germ-line embryos. Two genetically manipulated ES cell clones, which contained the *lacZ* reporter gene, were selected for this investigation, wherein one of these led to a very limited *lacZ* expression pattern (*Pax*5 +/- ES clone D3-15; Urbánek et al., 1994); whereas the other one

produced an extensive β-galactosidase staining (see below). Fig. 3 shows the comparison of *lacZ* gene expression in ES fetuses (Fig. 3A, C) with that in fetuses which originated from heterozygote crossings (Fig. 3B, D). E 9.5 embryos obtained from tetraploid blastocysts, which had been injected with the clone D3-15, showed a specific expression of the *lacZ* gene at the boundary between midbrain and hindbrain (Fig. 3A, arrow). This staining pattern was identical to that of embryos that were obtained from heterozygote crossings (Fig. 3B; see also Urbánek et al., 1994). The second ES clone that was investigated was an R1 clone of the designation R-βgeo3, which was obtained from a gene-trap experiment. R-βgeo3 cells were injected into diploid and tetraploid blastocysts. The injected diploid embryos produced fertile chimeras, some of which transmitted the *lacZ* transgene to their progeny. It took approximately four to five months to establish such a transgenic mouse line and to have available embryos from heterozygote crossings. Embryos, obtained by means of injection of R-βgeo3 cells into tetraploid blastocysts, were isolated on day 8.5 and stained for β-galactosidase activity. An intense staining was detected in the entire embryo itself, in the amniotic membrane (Fig. 3C, open arrow) and allantoin, but not in the yolk sac (Fig. 3C, arrow). This staining pattern was identical to the staining pattern in heterozygous embryos, which had been obtained according to heterozygote crossing (Fig. 3D). These results show that the expression pattern of the transgene remains reliably obtained in ES mice via germ-line transmission.

Table 1. Chimeric mice, produced from diploid embryos, which were aggregated or injected with wild-type ES cells

Cell line	Number of transferred embryos	Number of mice born	Number of chimeras M/F	Rate of chimera formation (range)	Germ line/tested
Aggregation: D3	15	3	2 2/0	2M=90.95%	1/1
Injection: D3	69	47	31 30/1	26M=95-100% 4M=80-90% 1F=90%	22/24 3/4 0/1
R1	24	14	12 7/5	7M=95-100% 5F=95-100%	5/5 3/4
J1	60	32	22 6/16	6M=95-100% 9F=95-100% 2F=80-90% 5F10-50%	6/6 3/8 1/2 2/5
GS1	15	4	4 4/0	4M=95-100%	4/4

M: males, F: females

Table 2. ES mice, produced from tetraploid embryos, which were aggregated or injected with modified ES cells

	ES clone	Number of passages	Number of transferred embryos	Living young in Caesarian section (proportion in %)	Number of surviving young (proportion in %)	Adult ES mice Number	M/F	Germ line/tested
Aggregation:	D3	p9	159	26 (16%)	0			
	J1	p11	37	1 (3%)	0			
	R1	p14	88	14 (16%)	5 (6%)	4	4/0	2/2
Injection:	D3	p9	69	19 (28%)	0			
	R1	p14	36	9 (25%)	5 (14%)	2	2/0	2/2
	GS1	p8	54	17 (31%)	6 (11%)	1	1/0	1/1
	R-169.2.3	p24	80	23 (29%)	12 (15%)	3	2/1	2/3
	R-*fra*3	p24	48	8 (17%)	5 (10%)	4	3/1	3/4

M: males; F: females

Table 3. Glucose phostate isomerase (GPI) analysis of ES mice

ES mice	Age	Number of mice	ES Cell contribution (%)												
			Liver	Lung	Heart	Intestine	Brain	Skin	Spleen	Stomach	Kidneys	Muscles	Testicles	Blood	Extremities
Aggregation:															
D3	E16.5	3	100	-	-	-	100	-	-	-	-	-	-	-	100
	E18.5	8	100	-	-	-	100	-	-	-	-	-	-	-	100
R1	E18.5	1	100	100	100	100	-	100	100	-	100	100	100	-	100
	E18.5	1	100	100	90	100	-	100	100	-	100	100	100	-	100
J1	E18.5	1	100	100	-	-	100	-	-	-	-	-	-		100
Injection:														-	
D3	E18.5	11	100	100	100	-	100	-	-	-	-	-	-	-	-
		1	70	80	90	-	100	-	-	-	-	-	-	-	-
		1	50	50	60	-	100	-	-	-	-	-	-	-	-
R1	E13.5	4	100	100	100	-	100	-	-	-	-	-	-	-	-
	E18.5	4	100	100	-	100	100	-	-	-	-	-	-	-	-
	adult	2	100	100	100	100	100	-	100	100	100	100	100	100	-
GS1	E18.5	8	100	100	100	100	100	100	-	-	-	-	-	-	-
	adult	1	100	100	100	100	100	-	100	100	100	100	100	100	-
MT*c-fos*LTR															
R-169.2.3	P3	1	100	100	-	100	100	100	-	-	100	-	-	-	-
	adult	3	100	100	100	100	100	-	100	100	100	100	100	100	-
*fra*1 +/-															
R-*fra*3	adult	1	100	100	100	100	100	-	100	100	100	100	100	100	-

E13.5, E16.5, or E18.5: 13.5, 16.5 or 18.6 days after beginning of pregnancy; P3: 3 days after birth.

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PATENT CLAIMS

1. Process for the production of mammals, which are derived from totipotent cells, with defined genetic properties, particularly transgenic mammals, in which totipotent cells of the same mammalian species are introduced into blastocysts and the obtained embryo is transferred to an asexual mother, characterized by the fact that totipotent cells with defined genetic properties are introduced into tetraploid blastocysts.

2. Process according to claim 1, further characterized in that the mammals are mice.

3. Process according to claim 1 or 2, further characterized in that blastocysts are used, which were obtained by means of electrofusion of two-cell embryos and subsequent cultivation.

4. Process according to one of claims 1-3, further characterized in that the totipotent cells are embryonic stem cells.

5. Process according to one of claims 1-3, further characterized in that the totipotent cells are embryonic germ cells.

6. Process according to one of the preceding claims, further characterized in that the totipotent cells are introduced into tetraploid blastocysts by means of microinjection.

7. Process according to one of claims 1 to 6, further characterized in that genetically manipulated totipotent cells are used.

8. Process according to claim 7, further characterized in that the genetically manipulated totipotent cells were obtained by introduction of plasmids, which bear the desired genetic modification.

Fig. 2
A. Newborn
lungs
thymus
salivary glands
skeletal muscle
eyes
brain
placenta
marker

skin
testicles
liver
spleen
pancreas
kidneys
heart
marker

B. Adult
male
female
male
female
marker